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DISCONTINUOUS RESPIRATION IN INSECTS AT LOW TEMPERATURES: INTRATRACHEAL PRESSURE CHANGES AND SPIRACULAR VALVE BEHAVIOR

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Insects face the problem of conserving body water. This problem is especially acute for pupae of the wild silkworm *Hyalophora cecropia* which in the northern parts of their range overwinter in environments where the temperature may drop to —30° C and below. At these low temperatures the humidity is also low, and there is a tendency for the animals to desiccate. Yet this rarely happens although pupae neither feed nor drink. Their only source of water, in addition to the body water with which they enter the pupal stage, is metabolic water.

Most pupal water loss occurs through the spiracles. When the spiracles open, air enters the tracheae and carbon dioxide and water vapor leave. Any mechanism that keeps the spiracles closed will enable the insect to retain water. Buck (1958) suggested that discontinuous respiration is such a mechanism.

Discontinuous respiration occurs cyclically; each cycle consists of a burst, a constriction period, and a flutter period which is followed by another burst, thus marking the beginning of another cycle. During the burst spiracular valves remain open for several minutes and then close for some time in the constriction period. Following the constriction period, valves begin opening and closing continuously or "fluttering," and this is the flutter period. Discontinuous respiration in Cecropia pupae is well-documented but evidence that these pupae exhibit discontinuous respiration at the low temperatures they experience is conflicting. Levy and Schneiderman (1966) reported cycles at 8.5° C, and Brockway (1964) recorded cycles at 0° C. However, Kanwisher (1966) concluded that there is no discontinuous respiration below 10° C and that pore diffusion accounts for gas exchange at low temperatures.

Understanding how these pupae conserve water at low temperatures and low humidities depends mostly on understanding how the spiracles behave under such conditions. This report examines the effects of low temperature on cyclical respiratory activity and on the behavior of spiracular valves.

Materials and Methods

Experimental animals

Diapausing pupae of *Hyalophora cecropia* had their brains removed three months before the experiment began to insure permanent diapause (Williams, 1946). Pupae then were stored at 22–25° C and 80–90% R.H. until used.

To record intratracheal pressure changes, the spiracles of pupae were cannulated

as described previously (Burkett and Schneiderman, 1974). The pupae then were placed in a water-saturated environment for two weeks to recover.

Recording intratracheal pressure changes

Intratracheal pressure changes were recorded continuously except during temperature equilibrations. The methods used were similar to those described in previous papers (Schneiderman and Schechter, 1966; Brockway and Schneiderman, 1967). Pressure changes as small as 0.025 mm Hg could be detected (Brockway and Schneiderman, 1967). As in these earlier papers it should be emphasized that the transducer repsonded to pressure changes in a system composed of the tracheal system, the cannula, and the transducer itself. Hence the recorded pressure changes were smaller than the actual intratracheal pressure changes that occurred in an intact pupa.

Recording spiracular valve behavior

Beckel (1958) described the anatomy of the spiracles of Cecropia in detail. The width of the spiracular opening is regulated by the spiracular valve which is moved by a closer muscle.

In one set of experiments reported here spiracular valve movements and intratracheal pressure changes were recorded simultaneously. To observe the valves of the third, fourth, and fifth right abdominal spiracles, they were exposed by scraping away the filter apparatus and gently pushing aside the peritreme with a hot needle. To keep the valves moist, a single transparent plastic window was sealed in place over them with melted paraffin. The valves were observed daily to insure that no sticking or desiccation had occurred.

The method described previously (Burkett and Schneiderman, 1974) for recording valve movements was used. Valve movements throughout at least one complete respiratory cycle were recorded at 20°, 15°, and 10° C. Below 10° C very long cycles precluded continuous direct observations and recordings of valve movements; thus 20-minute records with 20-minute intervals between recordings were made during the flutter and constriction periods of the cycle, and usually throughout the entire burst period. At — 10° to — 20° C valves were observed for 20-minute periods once every two hours.

Recording of intratracheal pressure changes at different temperatures

Intratracheal pressure changes of two pupae were studied by immersing the pupa-transducer system in a water-ethylene glycol bath. This method worked well at temperatures above 0° C. But at 0° C and below, recording of intratracheal pressure is affected noticeably by changes in room temperature and pressure. For this reason, and to permit observation of the spiracular valves at low temperatures, a different approach was developed. A pupa and the pressure transducer to which it was attached were placed in a small deep freezer fitted with a viewing window (Fig. 1).

The pupa and transducer were equilibrated at each temperature for 24 hours before recording was begun. Temperatures were lowered successively from + 25°

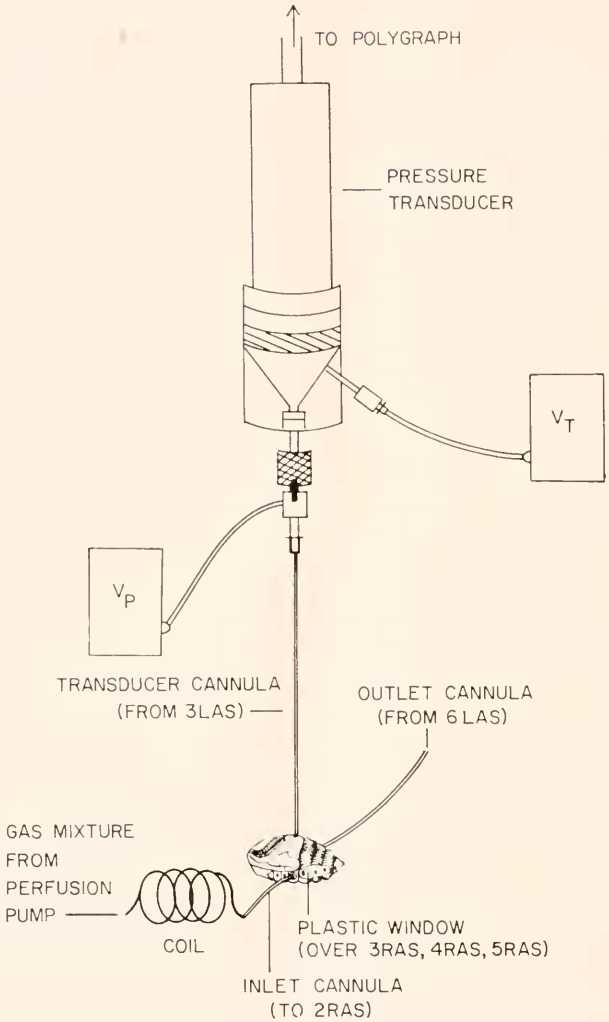

FIGURE 1. This assembly for simultaneously recording intratracheal pressure and valve movements was placed in a freezer, the temperature of which could be held constant to within $\pm 0.5°$ C at each experimental temperature between ambient and $-20°$ C. An infusion pump forced the experimental gas, cooled in the coil to the temperature of the freezer, into the tracheal system via the "inlet" cannula in the second right abdominal spiracle (2RAS). The "outlet" cannula in the sixth left abdominal spiracle (6LAS) prevented pressure build-up as a result of intratracheal perfusion. The cannula in the third left abdominal spiracle (3LAS) connected the tracheal system to the pressure transducer. To establish the baseline of intratracheal pressure records when the temperature was changed, and to check the size of a developing intratracheal vacuum without opening the freezer, the tracheal system was opened to the atmosphere by using two-way normally closed solenoid control valves (Allied Control Company, Plantsville, Connecticut). When the control valve of the transducer (V_t) was energized, fluid would have been forced from the transducer dome into the pupa had a second valve (V_p) not been used to equilibrate pressure. Movements of the third, fourth, and fifth right spiracular valves (3RAS, 4RAS, 5RAS) were observed and recorded as described earlier (Burkett and Schneiderman, 1974).

or + 20° C to −20° C in 5° intervals, and they were increased similarly to + 20° C after recording at − 20° C.

Intratracheal perfusions at low temperatures

In one series of experiments the responses of the spiracular valves to oxygen and carbon dioxide at low temperatures were examined. The composition of the intratracheal gas was regulated by perfusing known concentrations of oxygen and carbon dioxide through the tracheal system as described earlier (Burkett and Schneiderman, 1974).

TABLE I

Summary of data from records of intratracheal pressure and valve movements of pupa II at different temperatures

Temperature (°C)	No. of complete cycles recorded	Average length of cycle (min)	Average length of burst (min)	"Open" phase (min)	"Decline" phase (min)	Average length of constriction (min)	Average intratracheal vacuum developed during constriction (mm Hg)	Average length of flutter (min)	Average number of valve movements/min
+20	6	341	30 (8.8)*	21 (70)	9 (30)	41 (12.0)	−1.99	270 (79.2)	32
+15	6	365	72 (19.8)	59 (82)	13 (18)	56 (15.4)	−2.35	237 (64.8)	25
+10	3	706	85 (12.0)	45 (53)	40 (47)	104 (14.7)	−4.5	517 (71.8)	19
+5	6	839	85 (10)	34 (40)	51 (60)	377 (45)	−5.24	377 (45)	10
0	1(II) 2(III)	1517†	180† (11.9)	80† (44.4)	100† (55.6)	187(II) (12.3)	−3.13(II)	1229† (80.9)	1
−5	0	>16,200	Data incomplete	—	—	—	—	16,180	1/min-1/hr
−10	0	—	—	—	—	—	—	—	0
−15	0	—	—	—	—	—	—	—	0
−20	0	—	—	—	—	—	—	—	0

* Numbers in parentheses indicate per cent of cycle occupied by a given phase. Those for open and decline phases of burst are per cent of burst.

† Average of all such phases recorded from two pupae (II and III).

The experimental gas mixture was cooled to the temperature of the freezer before the mixture entered the tracheal system. Ten minutes were allowed for complete tracheal perfusion and equilibration with the gas before valve responses to the mixture were recorded. At least two hours were allowed for recovery from one gas mixture before a different mixture was introduced into the tracheal system. Air was perfused through the tracheal system during the recovery period.

Other procedures will be discussed in the appropriate sections below.

At least three cycles were recorded for each pupa at 5° intervals from + 20° to + 5° C. To record three complete cycles at 0° C two pupae wre used. No complete cycles were recorded below 0° C.

Results

Effects of decreasing temperatures on cyclical respiratory activity

Tables I, II, and III summarize data obtained from records of intratracheal pressure changes in one pupa. These data are representative of those obtained from all pupae used in these experiments. Figures 2, 3, and 4 show portions of the respiratory cycle at 0° and − 5° C.

The following changes in cyclical respiratory activity occurred as the ambient temperature decreased: (1) Discontinuous respiration persisted down to − 5° but not at − 10° C or below. (2) The duration of the respiratory cycle increased

Table II

Average length (seconds ± s. e.) of microcycles during different parts of the flutter period

Temperature (°C)	Part of flutter period		
	Beginning	Middle	End
20	57.4* ± 41.03** (10–160)†	58.53 ± 32.71 (20–134)	43.2 ± 16.24 (10–70)
15	54.83 ± 8.03 (10–120)	67.00 ± 27.07 (20–230)	47.4 ± 16.92 (10–80)
10	68.30 ± 47.84 (20–170)	76.33 ± 52.83 (20–200)	42.40 ± 24.77 (10–85)
5	202.97 (4–833)	229.73 ± 223.19 (20–820)	125.77 ± 109.39 (10–200)
0	452.43 ± 418.12 (20–1040)	809.57 (20–6864)	497.83 (30–3160)
−5	460 ± 423.72†† (60–7330)	—	—

* Each average figure is based on a total of 30 microcycles (ten from each of three cycles) from records of one pupa.

** S. D.

† Range.

†† Based on all microcycles recorded.

as the temperature decreased. Cycle length approximately doubled between 20° and 10° C, and between 10° and 0° C. (3) At temperatures above 0° C the pattern of the respiratory cycle was unchanged, that is, cycles were made up of a burst, during which valves remained fully open; a constriction period, during which valves remained closed; a pressure rise period following constriction, during which valves opened fully for several seconds and then closed again; and a flutter period, during which valves continuously opened briefly and then closed for several seconds. At 0° C, however, bursts and flutters persisted but constriction disappeared in some cycles. At − 5° C constriction disappeared altogther.

Changes in burst, constriction, and flutter

The percentage of a total cycle occupied by the burst did not change systematically as temperature decreased (Table I). The burst itself is made up of two distinct phases (Schneiderman, 1960): an "open" phase, during which

TABLE III

Average intratracheal vacuum (mm Hg ± s. e.) developed during microcycles in different parts of the flutter period

Temperature (°C)	Part of flutter period		
	Beginning	Middle	End
20	−0.113* ± 0.067** (−0.025 to −0.125)†	−0.096 ± 0.047 (−0.025 to −0.125)	−0.068 ± 0.041 (−0.025 to −0.050)
15	−0.089 ± 0.071 (−0.025 to −0.125)	−0.089 ± 0.045 (−0.025 to −0.150)	−0.080 (−0.025 to −0.050)
10	−0.188 ± 0.180 (−0.025 to −0.200)	−0.105 ± 0.075 (−0.025 to −0.250)	−0.067 ± 0.041 (−0.025 to −0.125)
5	−0.256 (−0.075 to −0.300)	−0.204 (−0.150 to −0.250)	−0.060 ± 0.037 (−0.025 to −0.150)
0	−0.260 ± 0.182 (−0.025 to −0.200)	−0.353 (−0.150 to −0.375)	−0.073 ± 0.049 (−0.025 to −0.150)
−5	−0.149†† (−0.04 to −2.273)		

* Each average figure is based on a total of 30 microcycles (ten from each of 3 cycles) from records of one pupa.

** S. D.

† Figures in parentheses indicate ranges of microcycles examined.

†† Based on all microcycles recorded.

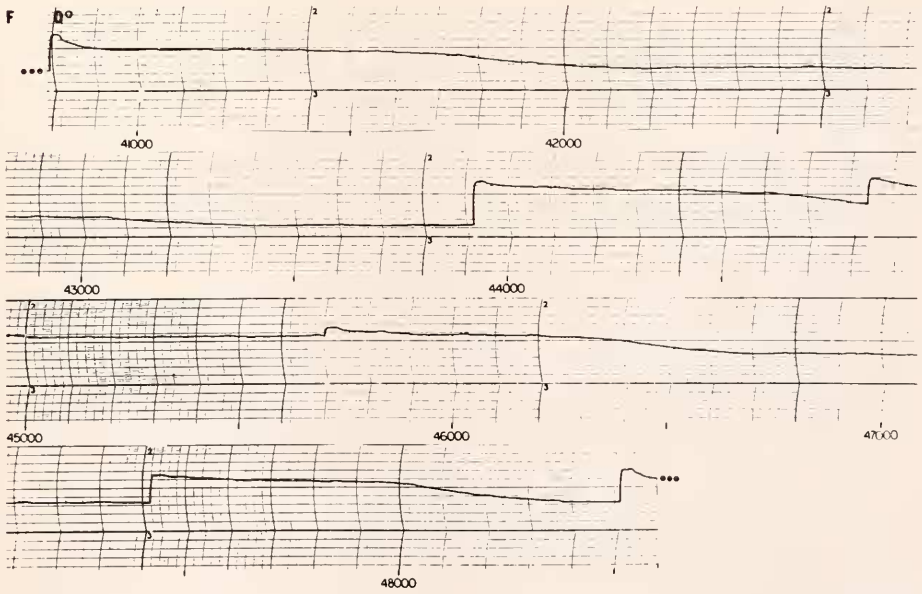

FIGURE 2. Intratracheal pressure record of pupa II at 0° C. Since this particular cycle lasted 28 hours, only a portion of the flutter period immediately preceding the burst is shown. The chart speed in this and subsequent records was 0.25 mm/sec unless otherwise indicated. Thus one small division between two thin vertical lines on the chart represents 20 seconds, and one large division between two heavy vertical lines represents 100 seconds. Recordings in this and other intratracheal pressure records were made at a sensitivity of 0.01 mV/cm.

valves are fully open and generally motionless; and a "decline" phase, during which valves oscillate about the fully open position. The percentage of the burst occupied by the decline phase increased as the temperature dropped below 10° C;

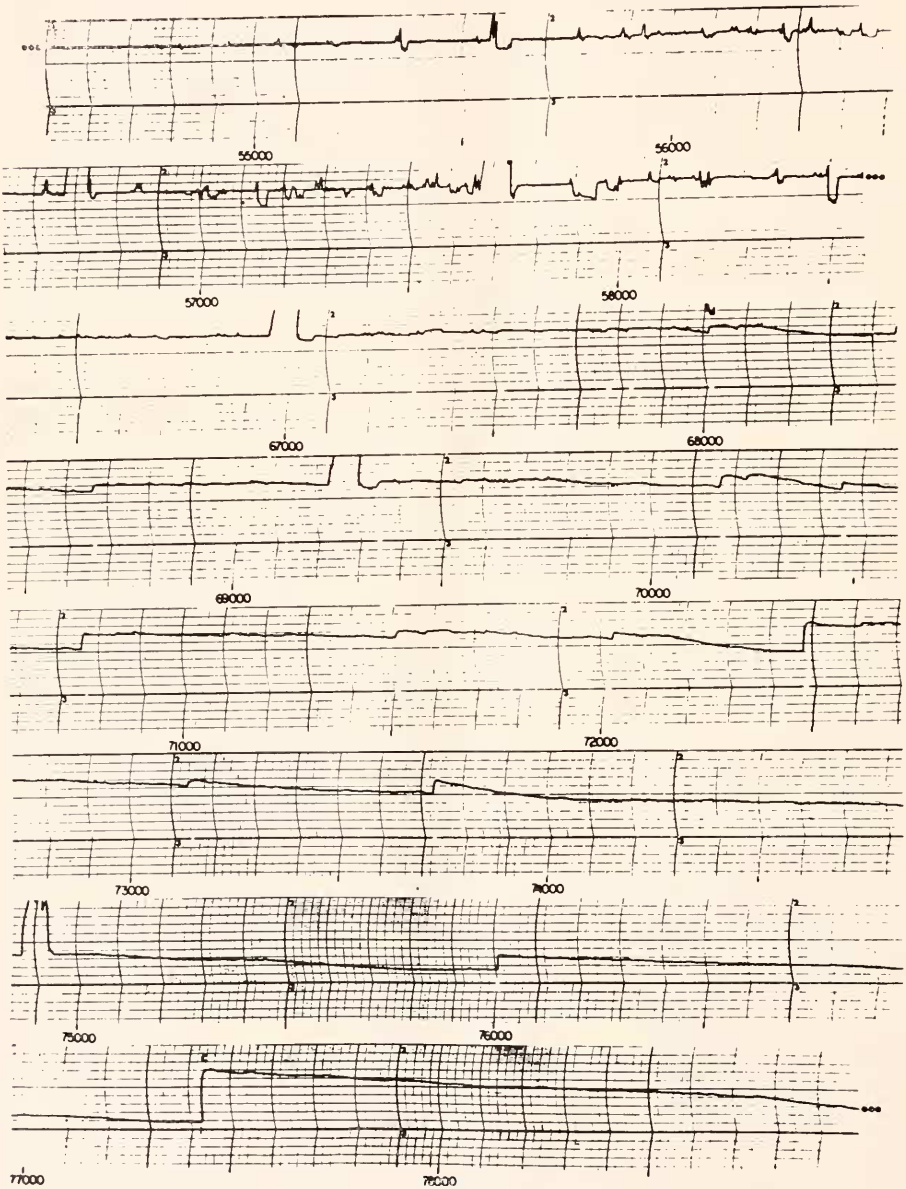

FIGURE 3. Intratracheal pressure record of pupa II during part of a burst at 0° C. A portion of the open phase (B_o) (54,500 to 68,000 seconds) and the entire decline phase (B_d) (68,000 to 77,400 seconds) are shown. Note the microcycles in the decline phase, for example, the one beginning at 76,000 seconds and ending at 77,420 seconds.

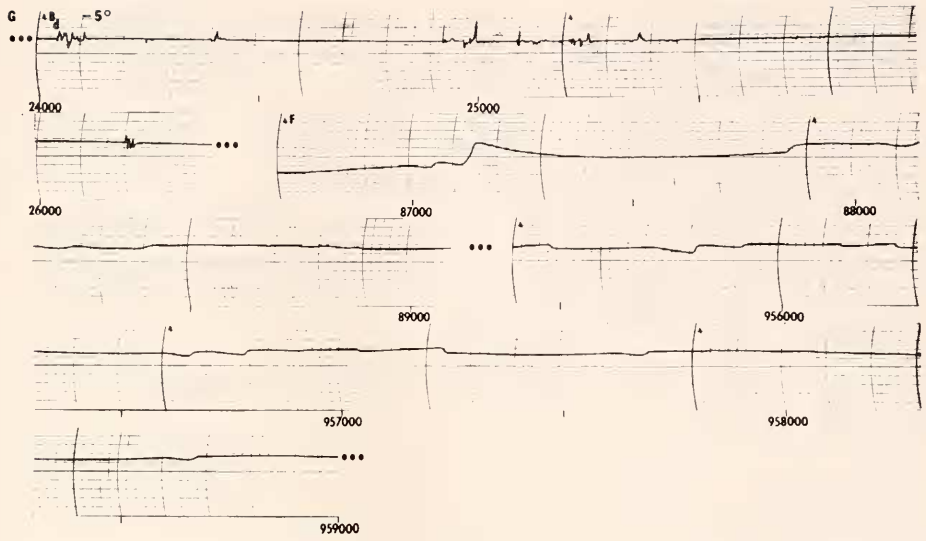

FIGURE 4. Intratracheal pressure record of pupa VI at −5° C. Since the cycle lasted at least 11 days, only part of the decline phase and a portion of the flutter period are shown. (Chart speed = 1 mm/sec.)

at 15° C it was 18%, whereas at 5° C it was 60% of the burst. At 15° C and below, the decline phase was marked by microcycles like those occurring during flutter.

The proportion of the cycle occupied by the constriction period did not change appreciably between 25° and 10° C, but at 5° C constriction was proportionally longer (45% of the cycle) than at other temperatures. The intratracheal vacuum that developed during constriction increased steadily from − 1.99 mm Hg at 20° C to − 5.24 mm Hg at 5° C, and then decreased to − 3.13 mm Hg at 0° C (Table I).

Above 0° C the flutter period occupied a greater proportion of the cycle than either the constriction period or the burst, except at 5° C where the flutter and the constriction periods were about equally long. In two cycles at 0° C the decline phase of the burst was followed not by constriction but by the flutter period; however, flutter was interrupted by periodic bursts. In another pupa the cycle at 0° C consisted of the usual burst, constriction, and flutter periods. Although no complete cycle was recorded at − 5° C, recordings of intratracheal pressure and valve behavior were begun during the early decline phase; like most cycles at 0° C, no constriction period was observed. The decline phase was followed by flutter which persisted for at least 11 days when the experiment was ended.

Changes in microcycles during the flutter period

Although valves open and close through a flutter period, they are closed most of the time (Schneiderman, 1960). When the valves open, intratracheal pressure rises to near atmospheric; when they close, intratracheal pressure falls. These

fluctuations in intratracheal pressure are microcycles. At 20° C an average microcycle lasted about 53 seconds but the duration varied from 10 to 160 seconds (Table II).

Table II shows that as the temperature decreased, the average duration of microcycles in the flutter period increased. There was wide variation in the duration of microcycles at a given temperature, particularly low temperatures. However, Table II shows that the longest microcycles occurred at the lowest experimental temperatures. For example, at − 5° C a microcycle lasting 7330 seconds (2 hours) was observed whereas at 20° C the longest microcycle was 160 seconds. On the other hand, relatively brief (60 seconds) microcycles occurred at − 5° C as well as at higher temperatures.

Microcycles occurring just before the onset of a burst appeared to be shorter and to result in a smaller intratracheal vacuum than those at the beginning or middle of the flutter period. To determine whether these differences were statistically significant, the duration and the size of the intratracheal vacuums in different parts of the flutter period were measured. The first ten microcycles at the beginning, the ten in the middle, and the last ten just before the onset of a burst were chosen in each of three cycles of one pupa at 5° intervals between 20° and 0° C. The results are shown in Tables II and III.

At a given temperature the length of microcycles was not significantly different (at the 0.05 level) in different parts of the flutter period. When the same parts of the flutter period were compared at different temperatures, there were no significant differences in the length of microcycles at 20° as compared with 15° C, or at 15° as compared with 10° C. However, below 10° C the duration of microcycles in a given part of the flutter period increased significantly between 10° and 5° C, and between 5° and 0° C.

The following general observations were made regarding intratracheal vacuums during flutter: (1) At a given temperature the vacuums occurring just before bursts were smaller than those at the beginning or in the middle of the flutter period. However, vacuums occurring before a burst at 0° C were not significantly larger than those at 20° C. (2) Vacuums occurring in microcycles at the beginning and in the middle of the flutter period were significantly larger at 0° than at 20° C. (3) There was a wide range in the sizes of intratracheal vacuums occurring during the flutter period at a given temperature; however, the largest vacuums during flutter occurred at low temperatures (Table III).

Effects of low temperatures on the behavior of spiracular valves

To determine how low temperatures affect the behavior of the spiracular valves, a pupa was exposed to different temperatures, and records of intratracheal pressure and movements of the third, fourth, and fifth right abdominal spiracular valves were made.

As the ambient temperature decreased, the average number of valve movements during the flutter period decreased from 32 per minute at 20° C to 1 per minute at 0° C (Table I). At − 5° C the number decreased even further, and sometimes valve movements occurred only once an hour. However, even at low temperatures there were periods when valves moved more frequently than on the average.

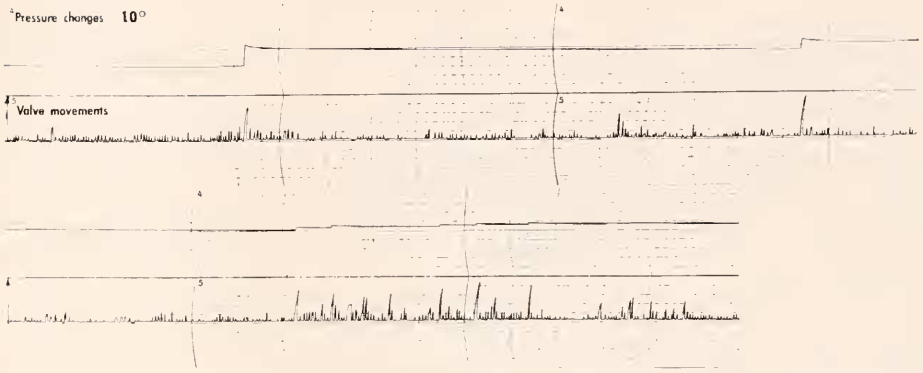

FIGURE 5. Simultaneous records of intratracheal pressure and behavior of 4RAS during the beginning of a pressure rise period at 10° C. Full opening of the valve is represented by a pen deflection of 5 divisions above baseline. (Chart speed = 1 mm/sec.)

Valves were capable of opening fully even at − 5° C but at this temperature opening often occurred so slowly that the movement could not be detected as it occurred. In this case movements were recorded only after it was obvious that the valve had moved.

Correlation of valve behavior with intratracheal pressure changes

Most pressure rises were accompanied by at least halfway opening of the fourth right abdominal spiracle, the spiracle we usually observed in all these experiments, although not every wide valve opening led to a pressure rise. Records of flutter occasionally showed that the valve of the fourth spiracle frequently began opening before a pressure rise occurred. However, many pressure rises were accompanied by, rather than preceded by, valve opening of the fourth right spiracle.

During the open phase of a burst at all temperatures, the valve of the fourth spiracle remained open except for occasional partial closures, so brief that the intratracheal pressure scarcely wavered from atmospheric. During the late portion of the decline phase of the burst, the intratracheal pressure records were very similar to those made during the flutter period; there were discrete microcycles, and the beginning of most microcycles was accompanied by wide opening of the valve of the fourth spiracle. However, not every wide opening was accompanied by a pressure rise.

During the constriction period, the valve of the fourth spiracle remained closed and motionless; at the same time the intratracheal pressure fell steadily. Constriction was followed by a step-wise pressure rise period (Fig. 5). As this figure shows, seven of the eight increments in pressure rise were accompanied by opening of the valve at least halfway.

Responses to oxygen and carbon dioxide

To determine what the spiracular valves do at temperatures below − 5° C, the following experiments were performed:

Ten pupae (5 per group) were weighed individually and then were placed immediately into one of two 650 ml desiccators, each containing a desiccant, $CaCl_2$ (Drierite) and a carbon dioxide absorbent, $Ca(OH)_2$ (Ascarite). Group I was kept at − 2° C and Group II at − 16° C for one week. At − 16° C the pupae were frozen and their blood was solid. Pupae then were removed individually from the desiccators and were reweighed. The average weight loss of pupae in Group I was 1.29 ± 0.56 (S.D.) % of the original weights; the average weight loss of pupae in Group II was $0.65 \pm 0.29\%$, or about half that of pupae in Group I. Since the spiracles are the major site of water loss, the valves of pupae at − 2° C must have been open more often than those of pupae at − 16° C.

The desiccator containing the Group II pupae then was flushed at room temperature with a mixture of 20% CO_2 + 80% air to open all spiracular valves of the pupae. The desiccator, now containing 20% CO_2, again was placed at − 16° C for one week. The average weight loss following this treatment was $1.44 \pm 0.65\%$, or 2.2× the average weight loss of the same pupae at − 16° C in air. When the spiracular valves were opened and the pupae subsequently frozen, pupal weight loss at low temperatures, *i.e.*, − 16° C, was comparable to pupal weight loss at higher temperatures, *i.e.*, − 2° C.

The average weight loss of two other groups of pupae (III and IV) in air at − 16° C was determined. Then, without permitting warming of either desiccator, the one containing Group III was flushed for 5 minutes with air while the desiccator containing Group IV was flushed simultaneously with 20% CO_2 + 80% air. Both desiccators were returned to − 16° C. When these pupae were reweighed at the end of one week, those in Group III had lost an average of $0.40 \pm 0.20\%$, and pupae in Group IV an average of $0.22 \pm 0.13\%$ of their weight of the previous week. Pupal weight loss, even in an atmosphere in which the carbon dioxide concentration was high, was comparable to weight loss in air at the same temperature (0.40% and 0.37% for Groups II and IV, respectively). Thus it appears that the spiracular muscle does not respond to high carbon dioxide concentrations at low temperatures (− 16° C). Possible explanations for failure of the valves to respond to carbon dioxide at low temperatures are considered in the Discussion.

Direct observations of the spiracular valves and recordings of movements of the fourth spiracle revealed the following: Valve movements occurred at all experimental temperatures between ± 20° and − 5° C. No movements were observed at − 10° C or below. When 20% CO_2 + 80% air was perfused through the tracheal system at temperatures between + 20° and 0°, the fourth spiracle and its adjacent controls opened fully within 5 minutes after perfusion was begun, and remained open and motionless for the duration of perfusion. At − 5° C these valves were about three-fourths open after 5 minutes of perfusion, but full valve opening could not be elicited at − 10° C or below even when 50% CO_2 + 50% air was perfused through the tracheal system for one hour. This result indicates that at − 5° C or below the spiracular muscle does not respond to carbon dioxide.

To determine whether the spiracular response at − 5° C is solely to carbon dioxide or if the spiracular mechanism also responds to oxygen, 20% CO_2 + 80% air was perfused through the tracheal system. Ten minutes after perfusion was begun, the valves were three-fourths open and motionless. At this point, with the valves still open, the temperature was lowered to − 10° C, and an air per-

fusion replaced the 20% CO_2 + 80% air flow. The air flow was continued for 17 hours with no change in the position of the valves. Nor did a perfusion of 100% O_2 for two hours elicit valve closure. Then the pupa, receiving an intratracheal air perfusion, was allowed to warm to − 5° C; about seven hours later the valves closed.

In a second approach to the same question, intratracheal perfusions of both 1 and 0.5% O_2 (balance N_2) elicited opening of the valves about 20 minutes after the flow was begun at − 5° C.

The spiracular mechanism is capable of responding to intratracheal oxygen and carbon dioxide at − 5° but not at − 10° C or lower.

Discussion

Results of the present studies show that cycles of discontinuous respiration in Cecropia pupae persist at −5° C but not at − 10° C. These results confirm and extend those of Brockway (1964) and Levy and Schneiderman (1966) but contradict those of Kanwisher (1966). Since discontinuous respiration depends on the responses of the spiracular nerve and muscle to oxygen and carbon dioxide, neuromuscular activity must also persist down to − 5° C.

As expected, the length of the respiratory cycle at low temperatures was greater than at high temperatures. At − 5° C, for example, the length of the cycle was at least 50 times greater than at 20° C even though two factors, a decrease in the CO_2-trigger threshold and an increase in the length of microcycles during the flutter period, tend to shorten the cycle. At low temperatures the CO_2-trigger threshold (the concentration of carbon dioxide that causes prolonged opening of all spiracular valves) decreases (Levy and Schneiderman, 1966), and this means that less carbon dioxide than usual is required to initiate a burst. On the other hand, an increase in the length of microcycles during the flutter period means that the proportion of the flutter period during which some out-diffusion of carbon dioxide can occur, *i.e.*, the pressure rise period of microcycles, is much less at 0° than 20° C. Brockway and Schneiderman (1967) estimated that if the average length of a microcycle were 25 seconds, the spiracular valves are open no more than 8% or, more likely, 5% of the flutter period. Since an average microcycle at 0° C may be 10 times as long as at 20° C, more carbon dioxide is retained at lower temperatures than at 20° C (*cf.* Schneiderman and Williams, 1955).

Since the length of the respiratory cycle increased at low temperatures, the combined effects of a reduced CO_2-trigger threshold and the increased length of microcycles clearly were offset by other factors. The key factor is that at low temperatures the metabolic rate of insects is lower than at high temperatures. Consequently, carbon dioxide production is depressed, and a longer time elapses before carbon dioxide reaches the CO_2-trigger threshold, reduced though it may be. In addition, more carbon dioxide dissolves in tissue fluids at low than at high temperatures, and this also increases the time required for carbon dioxide to reach the triggering threshold.

Significant changes occurred during the various phases of the cycle at low temperatures. One of the most noticeable changes occurred during the decline phase of the burst. Not only did the decline phase increase in length from an average of 9 minutes at 20° C to 51 minutes at 5° C (Table I) but also it

was detected from intratracheal pressure records at 15° C or below, but not at 25° or 20° C. A burst ends when the tracheal carbon dioxide falls to a minimum level (Schneiderman, 1960). Although tracheal oxygen quickly approaches ambient soon after a burst begins, the valves remain wide open for some time before they begin fluttering for an additional period (the decline phase), presumably because the pupa requires time to "unload" its accumulated carbon dioxide.

One possible explanation for prolonged valve openings during bursts at low temperatures is that the rate of carbon dioxide release is limited by the activity of carbonic anhydrase. Less than 15% of the carbon dioxide released during a burst by diapausing *Agapema* pupae is supplied by carbon dioxide in the tracheae at the onset of a burst (Buck and Keister, 1955, 1958), and this release occurs instantaneously. The remaining carbon dioxide escapes relatively slowly from the tissues during the remainder of the burst. There is enough carbonic anhydrase in the tissues of Cecropia pupae to account for the volume of carbon dioxide released during a burst (Buck and Friedman, 1958), and at low temperatures there is a decrease in the rate at which the enzyme acts. Thus, a pupa requires many minutes to unload carbon dioxide since the animal releases 2 to 3 times as much carbon dioxide during a burst at low temperatures as at higher temperatures (Schneiderman and Williams, 1955). Both the slow rate at which carbon dioxide is released and the larger volume of carbon dioxide that is impounded at low temperatures cause the valves to remain open for long periods.

There are three noteworthy points about the decline phase at low temperatures: (1) The initial part of the decline phase cannot be detected from intratracheal pressure records. (2) Fluttering and microcycles occur in later parts of the decline phase. (3) Most important, these flutters and microcycles, which constitute a second period of discontinuous respiration, persist even though the concentration of oxygen in the tracheae is close to ambient (20%). At first this is surprising since it is a *low* concentration of oxygen, *i.e.*, 5%, that normally triggers fluttering (Schneiderman, 1960; Burkett and Schneiderman, 1974).

Why do microcycles appear during the decline phase at a time when the tracheal concentration of oxygen is much higher than that which normally triggers fluttering? If carbon dioxide is indeed released slowly at low temperatures, the spiracles are still under the influence of carbon dioxide during the decline phase. The O_2-flutter threshold (the concentration of oxygen at which fluttering begins) can be raised by increasing the carbon dioxide concentration (Levy and Schneiderman, 1966; Burkett and Schneiderman, 1967, 1974). Thus fluttering can be induced even though the oxygen concentration in the tracheae is about 20% if the carbon dioxide concentration is about 7% (Burkett and Schneiderman, 1974), as it is during the decline phase (Levy and Schneiderman, 1966). Hence one expects fluttering during the decline phase.

Although the constriction period was proportionally longer at 5° C than at other temperatures (Table I), it disappeared in some cycles at 0° C and never was observed at − 5° C. Both the existence and the duration of the constriction period depend on the metabolic rate of the pupa. The size of the intratracheal vacuum, which was also greater during constriction at 5° C than at other temperatures, depends not only on the metabolic rate but also on changes in the volume of the tracheal system with intratracheal pressure changes, and on the

rate at which air leaks into the tracheal system (Schneiderman, 1960; Brockway and Schneiderman, 1967). At 5° C the metabolic rate of pupae still was high enough to cause them to use oxygen more rapidly than air leaked into the tracheae; as a result, a large intratracheal vacuum developed.

In two cycles at 0° C the decline phase led directly into a flutter period instead of the usual constriction period. The reason was this: Since the metabolic rate of the pupa was quite low, the rate at which the animal used oxygen was only slightly greater than that at which air leaked into the tracheae. Thus a significant vacuum, *i.e.*, one that would facilitate the mass transfer of air past the valves, could never develop. Eventually the intratracheal oxygen concentration dropped to the level that triggers fluttering and microcycles. The presence or absence of the constriction period at low temperatures depends on metabolic rate; pupae with a sufficiently high metabolic rate will have a constriction period, and those that have a lower metabolic rate will not.

The flutter period generally lengthened as the temperature decreased. This was as expected since the metabolic rate also decreased. However, at 5° C the flutter period and the constriction period were about the same length. A possible explanation for this is that the large intratracheal vacuums which developed during both the flutter and the constriction period at 5° C impeded out-diffusion of carbon dioxide; consequently, more carbon dioxide than usual accumulated. Thus the time required to reach the CO_2-trigger threshold decreased, and the flutter period was shortened.

The flutter period is a series of microcycles which generally became longer as the ambient temperature decreased (Table II). This, too, was expected since the duration of microcycles, like that of the constriction period, depends on the metabolic rate. When a pupa's metabolic rate is low, the rate at which it uses oxygen decreases. Thus a longer time is required for the concentration of oxygen in the tracheae to drop to the O_2-flutter threshold than when the metabolic rate is high.

In the present experiments the size of the intratracheal vacuum developed during flutter also increased except at − 5° C where it was less than at 0° C. During flutter at 25° and 20° C valves are constricted most of the time. At lower temperatures down to 0° C they are constricted even more since there are fewer openings per unit time than at higher temperatures. Consequently, larger vacuums can develop during the flutter period at lower than at higher temperatures. At − 5° C the average size of the intratracheal vacuum developed during flutter was less than at 0° C, presumably since the rate of oxygen uptake at − 5° C was so low that air leaked into the tracheae almost as rapidly as oxygen was removed.

During microcycles near the end of the flutter period the size of the intratracheal vacuum was less than in other parts of the flutter period (Table III). Observations of the spiracular valves explain why (Schneiderman, 1960). Before a burst, valves begin opening and closing more often and opening more widely than in other parts of the flutter period. As a result, intratracheal pressure never has an opportunity to fall much below atmospheric.

Most movements of the spiracular valves are not effective (Brockway and Schneiderman, 1967), that is, valves may move yet they may not open the tracheal system to the atmosphere or else they open it so briefly that no pressure

change occurs. The results of the present experiments confirm this. Changes in intratracheal pressure were accompanied by openings and closings of valves. However, most valve movements, especially at low temperatures, were pulsations—movements not strong enough to break the valve seal or to permit more than very small, very brief openings—and did not cause changes in intratracheal pressure.

Two factors must be remembered when attempting to correlate valve movements with intratracheal pressure changes: (1) If one spiracle closes, this does not necessarily mean that intratracheal pressure will fall since other valves may be open. (2) The fourth spiracle, which we observed, was covered with a plastic window; although the spiracle may have opened, it did not open the tracheal system to the atmosphere. The contralateral spiracle, which was not covered with a window, usually behaves in a similar manner but the precise movements of the two contralateral valves may not be exactly the same (Brockway and Schneiderman, 1967); in fact, about 20% of the time one of the two opens while the other may move slightly but does not open. Hence a brief opening in the fourth right spiracle may not have led to a synchronous pressure rise since the fourth left spiracle may not have opened at the same time. However, if the valve of the fourth right spiracle remained open for several seconds, other valves usually were also open.

There are several possible reasons why valves apparently do not respond to oxygen and carbon dioxide at temperatures below $-5°$ C: (1) The spiracular muscle itself may be insensitive to high carbon dioxide and to low oxygen concentrations. (2) The central nervous system, the principal target of oxygen (Burkett and Schneiderman, 1967, 1974) may not function; hence nervous control of spiracular activity may be blocked. (3) The enzymes that release the energy needed by a contracting muscle may not function at a level consistent with the energy requirements of the spiracular muscle (*cf.* Richards, 1958). (4) The muscle may be too viscous to move (*cf.* Richards, 1958). (5) The fluid film that covers the valves may become viscous, causing them to "stick." (6) The muscle may be frozen.

Which, if any, of these alternatives is responsible for the failure of the muscle to respond to oxygen and carbon dioxide is not known. However, the blood of pupae that were chilled to $-10°$ C under the conditions of our experiments (cooling rate $\leqslant 0.5°$ C/min) was frozen (Burkett and Schneiderman, 1968). This may also occur in nature.

The fact that discontinuous respiration and response of spiracles to oxygen and carbon dioxide were observed at $-5°$ C indicates that neuromuscular control of spiracular behavior persisted at this temperature. Although the spiracular muscle is innervated by a nerve from the central nervous system (Beckel, 1958), and is controlled in part by the nerve (Burkett and Schneiderman, 1974), Van der Kloot (1963) found that spontaneous potentials generated in the isolated spiracular muscle cause it to contract. He suggested that when the temperature is very low, it is these spontaneous potentials that keep the muscle contracted. Our results suggest that both the spiracular nerve and muscle are "turned off" at the same temperature or certainly within a few degrees of each other.

Fluttering is a significant mechanism by which diapausing pupae conserve water at low temperatures. Although the absolute duration of bursts at low temperatures

increases, there are fewer bursts than at high temperatures, and these two factors must offset each other in providing opportunities for pupae to lose water. In the present experiments not only did the flutter period lengthen as the temperature decreased, thus occupying most of the respiratory cycle, but also the number of valve movements during the flutter period decreased. These results support an earlier suggestion that ". . . the heart of the problem (water conservation) surely lies in the flutter period, where hour after hour the insect practices the 'trick' of filtering in oxygen, while retaining carbon dioxide and water" (Schneiderman, 1960, page 525).

At some temperature (between $-5°$ and $-10°$ C in the present experiments) discontinuous respiration ceases. At that point pupae must rely on other mechanisms of gas exchange and water conservation. Kanwisher (1966) concluded that at low temperatures, where discontinuous respiration no longer occurs, gas exchange occurs by pore diffusion. Since the pupa's respiratory demands are low ($\leqslant 0.1$ mm^3/g/hr at $-12°$ C (Kanwisher, as quoted in Asahina, 1966)), pore diffusion probably provides adequate gas exchange. Although less than 3% of the gas exchange at 25° C may occur through the cuticle (Schneiderman and Williams, 1955), at very low temperatures the insect's low metabolic demands may be met partially by this mechanism. But whatever the mechanism, one fact is clear: At low temperatures valves are constricted most of the time, and conditions in nature probably insure that they freeze in the constricted position. Hence the valves continue to serve the insect, enabling it to conserve water, even though they cannot respond to oxygen and carbon dioxide.

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Summary

1. Experiments were performed to examine the effects of temperatures down to $-20°$ C on discontinuous respiration in diapausing Cecropia pupae. Methods were developed that permitted simultaneous recording of intratracheal pressure changes and spiracular valve movements at low temperatures.

2. Discontinuous respiration continued down to $-5°$ C but ceased at some temperature between -5 and $-10°$ C.

3. At 0° C the constriction period of the cycle, during which valves remain closed for some time, generally was absent. The open phase of the burst was followed by a lengthy decline phase, during which valves close briefly from the fully open position, and then by the flutter period, during which valves continuously open briefly and then close. The flutter period made up most of the cycle at all temperatures except at 5° C where the flutter and constriction periods were of about equal length.

4. Longer microcycles, relatively brief pressure fall periods caused by the closing of the spiracular valves, with correspondingly greater intratracheal vacuums than at high temperatures were observed in the decline phase of the burst and in the flutter period as the ambient temperature decreased to 0° C. At $-5°$ C

microcycles were shorter and resulted in less of an intratracheal vacuum than at 0° C, presumably because the metabolic rate of the pupa decreased.

5. The spiracular valves responded to oxygen and carbon dioxide at − 5° C but not at − 10° C or below. Thus neuromuscular coordination of spiracular function persisted at − 5° C but ceased at some temperature between − 5° and − 10° C.

6. Studies of pupal weight loss indicated that spiracular valves remained constricted at low temperatures. Under the experimental conditions pupae froze at some temperature between − 5° and − 10° C. It is suggested that in nature the spiracular valves freeze in the closed position.

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